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Abstract

Rock glaciers (RG) are assumed to influence the biogeochemistry of downstream ecosystems because of the high ratio of rock:water in those systems, but no studies have considered the effects of a RG inflow on the microbial ecology of sediments in a downstream pond. An alpine RG-pond system, located in the NW Italian Alps has been chosen as a model, and Bacteria and Archaea 16S rRNA genes abundance, distribution and diversity have been assessed by qPCR and Illumina sequencing, coupled with geochemical analyses on sediments collected along a distance gradient from the RG inflow. RG surface material and neighbouring soil have been included in the analysis to better elucidate relationships among different habitats.

Our results showed that different habitats harboured different, well separated microbial assemblages. Across the pond, the main variations in community composition (e.g. Thaumarchaeota and Cyanobacteria relative abundance) and porewater geochemistry (pH, DOC, TDN and NH_4^+) were not directly linked to RG proximity, but to differences in water depth. Some microbial markers potentially linked to the presence of meltwater inputs from the RG have been recognised, although the RG seems to have a greater influence on the pond microbial communities due to its contribution in terms of sedimentary material.

Keywords

Rock glacier; alpine pond; sediments; microbial ecology; 16S rRNA

Introduction

Active rock glaciers (RGs) are slow-flowing mixtures of rocks and ice, representing one of the most common geomorphological features in high mountain environments [1], and are considered indicators of permafrost presence [2, 3]. Permafrost degradation has been reported to impact chemical characteristics of surface freshwater across the globe [4, 5] and RGs have shown the capability to influence waters passing through and originating from them, releasing cold meltwaters enriched in solutes [6-8]. Given the widespread distribution of RGs worldwide [1], the high concentrations of heavy metals [9] and nutrients [8, 10] in their meltwaters, and their lower ice-melting rate in comparison to glaciers [11, 12], their importance in shaping headwater biogeochemistry is probably going to increase in the future, in a climate change scenario.

Several studies (reviewed in [13]) have investigated how the presence of glacial-meltwater inputs can affect downstream freshwater ecosystems in terms of physical and chemical properties, with subsequent effects on their biological structure and function. Far less information is available on RG meltwaters, and particularly on their influence on the ecology of associated water bodies. For instance, Thies et al. [9] compared the diatom population of RG-influenced and reference streams in the Austrian Alps, highlighting that both diatom species composition and diversity can be negatively affected by the increase in water acidity commonly associated with RG runoff. In the NE Italian Alps, Ilyashuk et al. [14, 15] reported high concentrations of metals in the waters of RG lakes, which seem to explain the high rate of morphological abnormalities in chironomid population inhabiting lake sediments. Focusing on microbial community, Fegel et al. [8] described the bacterial community composition in stream sediments collected close to the terminus of several glaciers and RGs, distributed across three North America mountain ranges. They found that RG stream sediments were generally characterised by higher alpha diversity, and harboured a more cosmopolitan bacterial population, including genera more commonly found in soil. A similar approach was followed and similar results were obtained in the NE Italian Alps by Tolotti et al. [16]. However, to our best knowledge, the potential impact of RG meltwaters on both Bacteria and Archaea communities of a complex freshwater ecosystem has not been investigated yet.

In this study, we focused on a RG-pond system located in North-western Italian Alps, whose structural settings and hydrological dynamics have been previously described by Colombo et al. [17]. This pond represents for many reasons a suitable model system to investigate the influence of RGs on downstream aquatic ecosystems. The oligotrophic conditions, as well as the lack of algal or macrophyte cover on the bottom and the absence of direct anthropogenic disturbance linked to human activities carried out in the pond reduce the number of factors influencing the microbial community inhabiting sediments. Moreover, the limited dimensions potentially increase its susceptibility to external environmental pressures [18, 19]. The presence of a localised inflow from the RG has been confirmed [17], but it is important to specify that these meltwater inputs have been described as intermittent, concentrated only in the snow-free season and mainly linked to precipitation events [17, 20]. Meltwaters also showed only a moderate increase in solutes concentration if compared with the pond waters [20], differing in this sense from other RG-lake/stream systems affected by natural acid rock drainage phenomena [9, 14, 15]. Nevertheless, beside RG inputs can be limited in frequency and magnitude, microbial communities can quickly react to environmental pressures acting directly or indirectly on them [21], becoming a powerful instrument for the detection of ecosystem perturbations.

Our hypothesis was that the peculiar physicochemical properties of RG meltwaters and their potential to carry microorganisms of subglacial origin, may influence the sediments in terms of geochemistry, microbial abundance and microbial diversity. However, due to the intermittent nature and the limited magnitude of RG meltwater inputs, and to the presence of a localised inflow, we supposed that such an influence would not be equally extended over all the pond but would act mainly on the sediments closest to the RG inflow. Therefore, we hypothesized the occurrence of a horizontal gradient in RG influence, corresponding to a horizontal distance gradient from the RG inflow. In order to test this hypothesis, we firstly assessed archaeal and bacterial assemblages present in three main habitats (RG, surrounding soil and lacustrine sediments) of the RG-pond system. We than investigated whether the sediment microbial community and geochemistry varied across the pond, reflecting possible relationships with neighbouring compartments. Based on this information, we attempted to recognise microbial markers potentially symptomatic of the influence of RG on downstream pond sediments.

Materials and Methods

Study Site

The Col d'Olen Rock Glacier ("Corno Rosso 2 Rock Glacier" in the Aosta Valley rock glacier cadastre, http://geonavsct.partout.it/pub/GeoNavSCT/index.html?metadato=MTD010N0001) is a talus-tongue shaped RG [2], covering an area of about 37,500 m^2. It is located in the NW Italian Alps, at the boundary between the Aosta Valley and the Piedmont regions (45°52'8.22''N, 7°51'46.98''E, WGS84; Fig. 1a). The surface is covered by clasts (calcschists and serpentinites) varying from pluri-decimetric to metric size, with outcrops of fine-grained, serpentinitic material at the terminus and along the lateral scarps. The Col d'Olen Rock Glacier Pond is situated at the RG terminus, on the right side of the tongue (Fig. 1b), at an elevation of 2722 m a.s.l.. The pond has an area of 1,600 m^2, with maximum length and width of approximately 60 x 40 m, and reaches a maximum depth of about 3 m. It is a clear-water pond, characterised by oligotrophic conditions and by the lack of macroalgal and macrophyte cover on the bottom, with the shallower areas close to the shore undergoing seasonal freezing. More details on the catchment structural settings and hydrological dynamics of the pond are described in Colombo et al. [17, 20]. The research site is a node of the Long-Term Ecological Research (LTER) network in Italy (http://www.lteritalia.it).

Sampling Procedures

For this study, different typologies of sample were considered in order to assess the microbial assemblages present in the different habitats associated with the Col d'Olen RG (Fig. 1b-1c, Table 1). Sediment samples from the downstream pond were collected in triplicate at three points (S1, S2, S3) along a distance gradient starting from the RG front and crossing the pond in NE-SW direction. Points S1 and S3 were at approximately 1 m of water depth, while point S2 was positioned in the deepest point of the pond, at 3 m of water depth. The surface sediments (top 10 cm) were collected with a core sampler, transferred in sterile plastic jars without preserving sediments stratification, and kept covered with water during the transportation. Three aliquots of fine-grained serpentinitic material (top 10 cm) were collected on the RG front. A triplicate soil sample was collected from the North vegetated slope. Only the top 10 cm, after removal of vegetation and roots, were considered. All the samples were kept refrigerated and in the dark during the transport. In the laboratory, material for chemical analysis was stored at 4 °C, while aliquots for molecular analysis were preserved at – 20 °C prior to further processing.

The complete sampling procedure was repeated for two consecutive years, always during the advanced snow-free season (September 9th 2015, July 30th 2016), in order to avoid the additional effect of snowmelt processes on the system.

Samples Physicochemical Characterization

Pond sediments, RG material and soil

Solid samples were homogenized and sieved to 2 mm. A fresh aliquot of 20 g was extracted with 100 ml KCl 1M. Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were determined on 0.45 μm filtered extracts with a total organic carbon analyser (Vario TOC, Elementar, Hanau, Germany). Ammonium and nitrate concentration in KCl extracts were measured spectrophotometrically (U-2000, Hitachi, Tokyo, Japan). For extractable ammonia determination a Berthelot method based on reaction with salycilate in presence of alkaline sodium dichloroisocyanurate [22] was used while, for extractable nitrate, the Gneiss reaction [23] was applied. Total carbon (TOC) and nitrogen (TN) in the solids were determined on samples dried at 105°C by elemental analysis (NA2100, CE Instruments, Milan, Italy).

Porewater

Porewater was extracted from aliquots of fresh sediment by centrifugation and 0.20 μm filtered. pH was measured using a Basic 20 pH meter (Crison, Modena, Italy). Anions (NO_2^-, NO_3^-, Cl^-, SO_4^{2-}

and PO_4^{3-}) were analysed by ionic chromatography with a Dionex 500 (Thermo Scientific). Major cation (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) concentrations were measured by Atomic Absorption Spectrophotometry (Perkin Elmer AAnalyst 1400), while Si was determined spectrophotometrically according to standard methods [24].

DNA Extraction

Genomic DNA from sediments, soil and RG fine-grained material was extracted from 0.5 g of sample using the FastDNA™ SPIN Kit for Soil and the FastPrep® Instruments (MP Biomedicals) in accordance with the manufacturer's instructions. Extracted DNA was quantified using Qubit® (Life Technologies), quality was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific) and DNA integrity was evaluated by agarose gel electrophoresis.

Quantitative PCR Analysis

Quantification of 16S rRNA genes was performed by quantitative PCR (qPCR) using primer pairs 340F-1000R for Archaea [25] and 338F – 518R [26] for Bacteria. The reaction mixture contained 10 µl of SsoAdvanced™ SYBR® Green Supermix, 0.3 µM of each primer, and 2 µl of DNA diluted 1:10 (sediment, RG samples) or 1:100 (soil samples), in a total volume of 20 µl. Plasmids including a single copy of a single bacterial or archaeal 16S rRNA gene, amplified with the same primers used for qPCR, were serially diluted and used as standard for DNA quantification. qPCR reactions were performed using a Chromo4™ Real Time PCR Detection System (Bio-Rad Laboratories), and data were analysed with the MJ Opticon Monitor software (version 3.1). Both samples and standards were analysed in triplicate in PCR strip tubes (Bio-Rad Laboratories), under cycling conditions reported in detail in Online Resource 1. PCR specificity was verified by melting curves analysis and bands visualisation on agarose gel. Standard curves R^2 values were always higher than 0.997, and all the reactions showed efficiencies higher than 85%.

16S rRNA Gene Fragment Sequencing and Data Processing

The V4-V5 regions of bacterial and archaeal 16S rRNA genes were PCR-amplified using primer pair 515F-Y and 926R [27]. PCR reactions were performed in triplicate in a 25 µl reaction volume containing 1x KAPA HiFi HotStart Ready Mix, 0.2 µM forward and reverse barcoded primers and 12 ng of DNA. Cycling conditions were as described by Parada et al. and are resumed in Online Resource 1.

After the amplification, PCR products were checked for presence and intensity by electrophoresis on 1.5% agarose gel, and replicates of the same sample were pooled.
Amplicon purification, 16S library preparation and sequencing by MiSeq Illumina (Illumina, San Diego, CA) using a 2 x 300 bp paired-end protocol were performed at the Bristol Genomics Facility (Bristol, UK).

Raw Illumina reads quality was checked with Prinseq [28], and terminal portions with an average quality score below q15 across a 5 bp sliding window were trimmed. Forward and reverse priming regions were removed and sequences resulting shorter than 230 bp after the quality trimming were discarded. Paired-end reads were merged using FLASH [29] and processed sing QIIME pipeline [30]. Chimeric sequences were detected and removed using UCHIME2 in mode sensitive and Gold database as reference [31]. Sequences showing >97 % similarity were grouped in OTUs using uclust [32], and for every cluster the most abundant sequence was chosen as representative. The taxonomic affiliation of the OTUs was assessed by RDP classifier [33] against the SILVA 128 database [34] at a 97% confidence cut-off, and only representative sequences able to align to SILVA database were conserved and used for OTU table construction. Sequences appearing one time in a single sample were discarded, and one of the soil samples collected in 2015 (G3_15) excluded from the analysis due to the remarkably lower number of available sequences. OTU tables containing bacterial and archaeal sequences were obtained and analysed separately, and sequences deriving from mitochondria or chloroplasts were filtered from the bacterial OTU table.

Diversity and Statistical Analysis

Rarefaction curves were constructed without applying any normalisation on the number of sequences per sample, while for alpha and beta diversity evaluation, the number of sequences per sample was normalised according to the less abundant sample. Alpha diversity estimators (number of OTUs, Chao1, Shannon and Simpson indexes) were calculated on multiple rarefied OTU tables using QIIME [30]. The same program was used to align bacterial or archaea OTU sequences [35], to build the corresponding phylogenetic trees [36], and to compute Bray-Curtis, Weighted and Unweighted UniFrac distance matrixes [37] based on similarities at OTU level. Ordination of samples according to the different distance matrixes was performed by non-metric multidimensional scaling (NMDS), and the significance of the separation among environmental matrixes and sampling points was evaluated by PERMANOVA (permutational multivariate analysis of variance) using the adonis function of the ‘vegan' package in R [38].

In order to better elucidate taxon-habitat relationships, an indicator species analysis was performed. Archaeal and bacterial OTUs showing relative abundance higher than 0.1% were analysed separately. The number of OTUs per sample was normalised based on the less abundant sample by using the rrarefy function of the 'vegan' package. IndVal statistics were calculated with the multipatt function (with 99,999 permutations) of the 'indicspecies' package in R [39]. P-values were corrected for multiple testing according to the FDR procedure, and only associations with PFDR > 0.05 were retained as significant. Moreover, in order to focus on the strongest associations, only OTUs showing IndVal values higher than 0.90 were considered. Indicator OTUs were then grouped into phyla/classes and their association with different habitats were represented in bipartite networks by using the Gephi software [40].

Geochemical data, 16S rRNA genes abundance, alpha diversity estimators and the relative abundance of the most abundant bacterial and archaeal classes were compared among the different sampling points, by analysing separately 2015 and 2016 datasets. Data were transformed if required, normal distribution of residuals and homogeneity of variances were tested and, when appropriate, ANOVA was applied. In presence of significant differences, pairwise comparisons were performed using Tukey's honest significance difference (HSD) test, and differences were considered significant for $P < 0.05$. When ANOVA assumptions were violated, Kruskall-Wallis nonparametric test coupled with Dunn's test as *post hoc* was applied. In this case, P-values were adjusted for multiple comparisons by Holm-Šidák method.

All the plots (diversity bar plots, boxplots and NMDS plots) were drawn with 'ggplot2' package [41].

Data Accessibility

Raw sequencing data were deposited in NCBI SRA database under accession number SRP126235.

Results

Sediments, Soils and Porewater Geochemistry

All the considered geochemical parameters showed a certain degree of variation in relation to the environmental matrix (Fig. 2, Online Resource 3). Total C and N, as well as DOC and TDN were significantly lower in RG samples, intermediate in the pond sediments and higher in soil (G). RG samples were also characterised by the lowest levels of ammonia, while the highest levels were

detected in sediment samples. The C/N ratio in RG was significantly higher than in sediments and soil. For nitrates, homogeneous values were reported for all the samples but G, RG and S3 in 2015.

Porewater characterisation of the lacustrine sediments is reported in Table 2. When comparing sediments collected at different sampling points (Fig. 2, Online Resource 3), several geochemical properties appeared to follow a similar trend in both the sampling times. For instance, DOC, TDN, NH_4^+ concentrations and porewater pH were higher in the central, deepest samples (S2), and lower in S1 and S3.

Bacterial and Archaeal 16S Abundance

16S rRNA genes abundance showed the lowest values in RG samples, with an average of 9.33 ± 0.24 and 7.51 ± 0.07 Log copies per g of dry weight for Bacteria and Archaea, respectively (Fig. 2, Online Resource 3). Soil was characterized by higher abundances: between 9.96 and 11.15 Log copies per g of dry weight for Bacteria, and between 7.99 and 8.88 Log copies per g of dry weight for Archaea. A similar range in 16S genes abundance was also reported for pond sediments, with bacterial abundance ranging between 9.64 and 11.01 Log copies per g of dry weight, and Archaea between 6.97 and 8.89 Log copies per g of dry weight. In general, in all the environmental matrixes 16S bacterial markers outnumbered their Archaea counterpart by at least 1 or 2 orders of magnitude.

Bacterial and Archaeal Community Diversity

A total of 6,131,017 high-quality sequences were obtained after trimming and filtering procedures. 96.5 % of the sequences was classified as Bacteria (63,962 OTUs), 3.5 % as Archaea (2478 OTUs), and a negligible number remained unclassified at domain level.

Analysis of rarefaction curves (Online Resource 2) confirmed that a satisfactory level of coverage was achieved for bacterial sequences (with Good's Coverage estimator values higher than 94%) in all the samples, while lower coverage values were reported in sediment samples for archaea (average 80%). This is coherent with trends in alpha diversity estimators (Fig. 3, Online Resource 3) applied to the archaeal community, showing a decrease in richness (OTUs number, Chao1 index), Shannon and Simpson diversity indexes from sediment to soil, to RG communities. However, comparing between sediment samples only, lower diversity characterized samples S3, particularly in 2015. For Bacteria, shallow sediments (S1, S3) showed higher Chao1 values if compared with RG and G samples, but they did not differ significantly in terms of observed OTUs,

Shannon and Simpson indexes. Conversely, sample S2 always reported lower richness and diversity relative to all the other samples.

For beta diversity, NMDS ordination plots based on different distance estimators all showed a strong separation among communities inhabiting different environmental matrixes, while no apparent differences in terms of sampling year were detectable (Fig. 4). In particular, for archaeal community, Bray-Curtis and Unweighted UniFrac distances defined a sharp separation among pond sediment, RG and soil samples, while Weighted UniFrac suggested higher similarity between soil and RG communities. For bacteria, all the tested distances produced separate clusters corresponding to different sample typology and were also able to divide shallower sediment samples (S1, S3) from the deeper sediment sample S2. PERMANOVA analysis on Bray-Curtis, Unweighted and Weighted UniFrac distance matrixes confirmed the presence of significant differences (Pseudo-$F_{(4,28)} \geq 8.0$, $P < 0.001$; Online Resource 3) in archaeal and bacterial community structure among different environmental matrixes.

Composition of Archaeal and Bacterial Communities

The taxonomic composition of the archaeal community was clearly differentiated based on sample type (Fig 5a, Online Resource 4). Terrestrial ecosystems were dominated by Thaumarchaeota, representing approximately 90 % and 80 % of RG and G sequences respectively. However, while RG surface material community included mainly members of the Soil Crenarchaeotic Group (SCG), a pool of additional classes (e.g. FHMa11terrestrialgroup, SAGMCG-1) was also present in the soil samples. Moreover, by examining in detail the sequences affiliated with SCG, a separation between RG and soil samples in terms of dominant OTUs emerged. Thaumarchaeota were also detected in sediment samples, but only represented by the Marine Group I class, with one dominant OTU identified as Cand. *Nitrosoarchaeum* accounting for 80-99% of total Thaumarchaeota sequences.

The most represented phyla in the pond sediment samples were Woesearchaeota (49-63 % of sequences per sample) and Euryarchaeota (16-38 %), the latter including mainly Thermoplasmata and Methanomicrobia classes. Despite the presence of Thermoplasmata in all the samples, communities associated to different environmental matrixes showed a clear separation at family level.

For bacterial community (Fig. 5b, Online Resource 4), all the samples across all habitats harboured essentially the same classes, but variations in their relative abundance distinguished communities belonging to different environmental matrixes. In particular, soil community differed

from RG and sediment community due to significantly higher proportions of Alphaproteobacteria, Acidobacteria (Acidobacteria, Solibacteres) and Planctomycetes (Planctomycetacia) and lower proportions of Betaproteobacteria (including mainly Burkholderiales, Nitrosomonadales and SC-I-84 classes). Distinctive features were also detectable by comparing RG and sediment samples: sediment community was characterised by higher Cyanobacteria, Deltaproteobacteria and Ignavibacteria relative abundances, while RG samples showed higher proportions of Actinobacteria (Thermoleophilia, Actinobacteria belonging to Frankiales, Micrococcales and Propionibacteriales), Acidobacteria (Blastocatellia) and Bacteroidetes (Sphingobacteriia).

By comparing only sediment samples, some microbial groups appeared to follow peculiar trends. Cyanobacteria proportion, for instance, appeared to be higher in central (S2) relative to shallower sediment samples (S1, S3), showing also a differentiation in terms of dominant OTUs, while an opposite trend was reported for Thaumarchaeota and Acidobacteria. Moreover, some classes (e.g. Gemmatimonadetes, Blastocatellia) exhibited a reduction in percent abundance along a distance gradient moving from the RG front.

Finally, samples S3 collected in 2015 displayed anomalous characteristics in terms of community composition, if compared with all other sediment samples, including the three collected in the same point in 2016. For instance, they harboured lower proportions of Bathyarchaeota and Methanomicrobia, and higher proportions of Thermoplasmata (dominated by a single OTU) and Betaproteobacteria belonging to the Oxalobacteraceae family.

Indicator Species Analysis

A total of 55 and 780 OTUs significantly associated to a single sampling point or to a combination of sampling points was retrieved for Archaea and Bacteria, respectively (Online Resource 5).

In the case of Archaea, the analysis showed (Fig. 6a) a sharp separation between indicator OTUs associated with terrestrial and lacustrine habitats, without indicator OTUs shared between the two groups. In the pond sediments, the highest number of indicator OTUs was reported for the sampling point S2 (12 OTUs), followed by the combination S1+S2 (7 OTUs).

A more complex network of associations among different sampling points and habitats was reported when considering bacterial OTUs (Fig. 6b). A considerable number of indicator OTUs was found to characterise the different habitats (G = 121; RG = 103; S1+S2+S3 = 163). Investigating the relationships between the sediments (S1+S2+S3) and the terrestrial habitats, the highest number of indicator OTUs was found to be shared with RG (69 OTUs), while only 8 OTUs were shared

with G, and no OTUs were shared with both RG+G. Moreover, comparing different groups of sediment samples, the combination S1+S3 was the one with the highest number of indicators shared with all the terrestrial habitats. Finally, focusing on single sediment samples, sampling point S3 showed the highest number of indicator OTUs shared with both RG (24 OTUs) and the combination RG+G (10 OTUs). The majority of them was closely related to indicator OTUs found for the S1+S2+S3 and S1+S2+S3+RG combinations, but OTUs affiliated to Intrasporangiaceae and Methylophilaceae were exclusively found to be representative of the combination S3+RG (Online Resource 5).

Discussion

Despite the increasing interest on the effects of RG thawing, only few studies have considered its consequences on the ecology of downstream ecosystems [8, 9, 14, 15]. In this study, we sampled different compartments of an alpine RG-pond system to: 1) investigate their microbial community structure and identify the key microbial groups that characterise each habitat; 2) assess the potential links between microbial diversity and geochemistry; 3) propose microbial markers potentially indicating the presence of RG influence on connected freshwater ecosystem.

Microbial Community Structure in the Different Habitats of an Alpine RG-Pond System

In general, beta diversity analysis highlighted a sharp separation among communities associated with different environmental matrixes, for both archaea and bacteria.

Soil bacterial community was dominated by Acidobacteria and Proteobacteria, followed by Planctomycetes, Chloroflexi, Actinobacteria and Verrucomicrobia, as previously reported for several alpine and high latitude tundra soils [42-44]. However, while these studies included Cyanobacteria among the most represented phyla, in our case cyanobacterial sequences represented less than 1% of the total soil sequences. The archaeal community from soil also corroborated with other studies where a prevalence of Thaumarchaeota sequences, all affiliated with terrestrial groups, was found, followed by Euryarchaeota of the class Thermoplasmata [45-47].

Considering lacustrine sediments, a recent survey on 13 freshwater lakes of the Yunnan Plateau (southwestern China) highlighted that bacterial community composition can remarkably vary in terms of dominant phyla among different high elevation freshwater lakes, although factors governing the intra-lake distribution of sediment bacterial and archaeal communities remain unclear [48]. However, some common traits appear to be conserved among the abovementioned lakes and

386 the Col d'Olen Rock Glacier pond bacterial community structure, with Proteobacteria
387 (Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria), Planctomycetes (in Olen
388 sediments mainly Phycisphaerae and Planctomycetacia) and Chloroflexi being the dominant groups.
389 Nevertheless, other phyla often poorly represented in the Zhang et al. study, like Nitrospira,
390 Gemmatimonadetes and Ignavibacteria had higher relative abundances in the Col d'Olen Rock
391 Glacier pond.
392 Freshwater ecosystems have been reported to harbour, on average, more rich and diverse archaeal
393 communities if compared with soil [49], and this was also the case of Olen pond system, and
394 differences in dominant archaeal taxonomic groups among alpine lacustrine ecosystems exist. For
395 instance, sediment communities dominated by Bathyarchaeota and methanogenic Euryarchaeota
396 (Methanomicrobia, Methanobacteria and Thermoplasmata classes) have been described in lakes of
397 the Yunnan and Tibetan Plateau in China [48, 50, 51], or in a number of water systems in the
398 Iberian Peninsula [52, 53]. However, even if 16-38% of total archaea sequences affiliated to
399 Euryarchaeota (mainly Methanomicrobia), Olen pond showed a quite different situation, with only
400 small proportions of Bathyarchaeota (0.2-4%) and a prevalence of Woesarchaeota (49-63%).
401 Analogous archaeal community composition has previously been described in waters of high-
402 altitude Pyrenean lakes [54].
403 The RG fine-grained surface material offered the harshest conditions for life of all the
404 investigated compartments, probably due to a combination of physical instability and lack of
405 vegetation cover. The low TOC and TN and the high C/N ratio, potentially indicating limited rates
406 of mineralization, seem to confirm the impact of such environmental constraints on resident
407 microbial communities. However, these challenging conditions seem to influence in different ways
408 Bacteria and Archaea. Bacterial abundance was significantly lower in the RG samples than in soil
409 or sediments, but community richness and evenness were comparable to the other studied habitats.
410 Conversely, for Archaea no significant differences were detected between RG and sediments for the
411 16S copy number, but alpha diversity was lower in RG than in sediments. This might suggest the
412 presence of a less diverse but highly specialized archaeal population, able to proficiently colonise
413 the RG surface. For instance, Thermoplasmata in RG samples included mainly representatives of
414 Marine Group II plus two highly abundant unidentified OTUs, while soil was dominated by
415 sequences of the Terrestrial Miscellaneous Group (TMEG) and sediments showed more complex
416 assemblages. This is further corroborated by the indicator species analysis.
417 The comparison of the bacterial community between different habitats showed that the RG
418 community was more closely related to sediment than soil, where proportions of phyla typically

associated to terrestrial habitats like Acidobacteria, Actinobacteria and Alphaproteobacteria were highly represented. If the separation between RG and soil communities may be partially attributed to differences in lithology (serpentine vs calcschist), as demonstrated in other alpine, cryoturbated soils [55], the different stage of habitat evolution is also likely to play an important role. The presence of Sphingobacteriales, Actinobacteriaceae affiliated to Micrococcales and Propionibacteriales, and Comamonadaceae in the RG matrix resembled other high elevation, poorly evolved soil ecosystems, such as unvegetated glacier forefields [56] and debris-covered glaciers [57].

Trends in Geochemistry and Microbial Community Structure

In this study, we also aimed to describe variations in microbial community composition and abundance across the pond, in relation to sediment geochemistry. The choice of only three sampling points at two different depths in the pond system and the focus on just one sampling time in the snow-free season, albeit repeated for two consecutive years, prevented detailed definition and verification of temporal, horizontal and vertical trends. However, the obtained data still offered some insights on the overall ecosystem relations between geochemistry and microbial community composition. For instance, our initial hypothesis was that the presence of RG hydrological inputs into the pond documented by Colombo et al. [17, 20], although limited in time and magnitude, would have led to a differentiation of sediments closest to the RG front from the farther ones. Instead, we found out that the strongest differences in terms of both geochemistry and microbial diversity exist between shallow (S1, S3) and deep (S2) sediments, regardless their distance from the RG front, and this trend seems to be conserved for the two seasons covered by this study. Water depth has been previously reported as one of the factors involved in community shaping in lacustrine and marine sediments [58], but it is interesting to find similar significant differences at a small scale (2 m of variation in water depth, about 30 m of horizontal distance), in a well-mixed, not stratified lacustrine ecosystem. Among the Archaea, the reported separation pond centre-borders is limited to the Thaumarchaeota phylum, which showed higher relative abundance in shallow than deep sediments. The absolute prevalence in sediments of sequences affiliated to Marine Group I and identified as Candidatus *Nitrosoarchaeum*, commonly found in freshwater ecosystems [52, 59], excludes the hypothesis of an accumulation of microorganisms or DNA from surrounding compartments (richer in Thaumarchaeota sequences, but mainly affiliated to others classes), suggesting that factors regulating their distribution must depend on lake intrinsic dynamics. This is also in accordance with the absence of indicator OTUs shared between any pond sediment and G or

RG samples. Another picture of intra-pond variation is offered by the analysis of the bacterial community structure and composition. On the one hand, the shallow sediment samples were clearly separated from the deepest ones in terms of overall community structure (as shown by NMDS analysis based on both phylogenetic and not phylogenetic distances) and showed the highest number of indicator OTUs shared with G and RG samples, potentially indicating stronger relationships with the terrestrial habitats. On the other hand, the deepest samples harboured higher proportions of cyanobacterial sequences than the shallow ones, mainly corresponding to a single OTU showing 100% sequence similarity with *Cyanobium* species and uncultured Cyanobacteria detected and isolated from the Baltic Sea [60] and lacustrine sediments [61]. Interestingly, the relative abundance of such OTU decreases in shallower sediments, where other phylotypes occupy higher proportions of the cyanobacterial sequences pool. The presence and activity of Cyanobacteria in marine and lacustrine sediments at depths far higher than 3 m, where the penetration of solar radiation is limited, has been widely reported in literature [62-65]. Moreover, several studies described how microalgal and cyanobacterial community structure and diversity in freshwater lake sediments vary in relation to water depth, often as a consequence of disturbance events affecting shallow sediments, i.e. drying-rewetting or exposure to freezing [66-68]. Therefore, the variations observed in Olen Pond in terms of Cyanobacteria relative abundance and community composition may be consistent with the potentially more stable conditions offered throughout the year by the pond deepest zone, apparently not affected by water freezing phenomena that regularly occur in the lateral, shallower areas.

Microbial Markers and Localised Anomalies in Alpine RG-Pond Systems

In the last part of this study, we have considered possible candidates for microbial markers that can be used to indicate the presence of RG inputs in a connected alpine freshwater ecosystem. Both the indicator species analysis and the community composition data showed the existence of a stronger link between sediment and RG habitat than between sediment and soil, at least for the bacterial community. However, when formulating our hypothesis, we pointed out that the hydrochemical influence of the RG on the overall Olen pond is limited in frequency and magnitude and, as demonstrated by previous studies [17, 20], it is not apparently affecting the entire pond in terms of hydrology and water chemistry. Therefore, a different explanation for the links between pond sediment and RG bacterial communities should be looked for, and to considerate the issue under a geomorphological point of view may be useful in this sense. From an exclusively geomorphological standpoint, RGs constitute prominent sedimentary linkages within the alpine

485 environment, deeply contributing to alpine erosion [69]. Although they are chiefly considered one
486 of the main storage components of the coarse debris system [70], the amount of fine-grained
487 material in the RG interior can be relevant (Haeberli et al., 2006), as also observed in the frontal and
488 lateral scarps of the Col d'Olen Rock Glacier. Thus, since the RG offers a proportion of mobile
489 fine-grained material (cf. [71]) far higher than the surrounding vegetated soils, undergoing slower
490 rates of erosion (cf. [72]), we can expect to find an accumulation of such material on the bottom of
491 the pond in which the RG front is ending. As previously mentioned, the lithological characteristics
492 of the substrate are known to strongly influence microbial communities structure and composition
493 [73-75], thus the occurrence of RG sedimentary deposits in the pond may explain the analogies in
494 the dominant bacterial phyla/classes proportions and indicator OTUs between pond sediments and
495 RG samples.
496 Indicator analyses on the sampling point closest to the RG inflow (S3), where we expected
497 to detect a possible effect of RG hydrochemical contribution on the sediment communities, showed
498 a higher number of indicator OTUs shared with RG in comparison to the other sediment samples.
499 Nevertheless, the presence of OTUs closely related to phylotypes characterising the overall
500 sediment habitat or the combination sediments + RG did not allow to clearly confirm the link
501 between their occurrence and the presence of RG meltwater inputs.
502 In this study we also detected anomalies in the S3 samples collected in 2015, that could be
503 interpreted simply as a product of random interannual variability, but might also suggest that in
504 some circumstances the RG hydrochemical influence can be enhanced and become apparent.
505 Considering purely physical and chemical data, the waters in point S3 between mid-August and
506 mid-September 2015 were clearly differentiated from all the other sampling points across the pond
507 with colder water temperatures, higher electrical conductivity and higher solutes (including nitrate)
508 concentration. Colombo et al. [17, 20] attributed this evidence to a phase of high RG hydrological
509 contribution. Concerning the anomalies described in this study for samples S3_15, many of them
510 are in accordance with the physical and chemical observations. The higher nitrate concentrations in
511 sediments are coherent with those measured in waters. The increase in the relative abundance of
512 Thermoplasmata, with around 96% of sequences corresponding to a single unidentified OTU
513 present in lower proportions in all the other pond sediments samples, as well as the lower archaeal
514 diversity, are consistent with the possibility of a localised selection process, directly or indirectly
515 driven by RG influence. Finally, the higher relative abundance of Oxlobacteraceae, belonging to the
516 order Burkholderiales, that include many chemolithotrophic and autotrophic microorganisms

517 frequently associated with glacial and subglacial environments [57, 76], is in line with the
518 hypothesis of a localised, meltwater-driven microbial enrichment.
519 The absence of similar characteristics in the sediment samples S3 collected in 2016 could be
520 linked to the sampling, performed earlier in the snow-free season in comparison to 2015. Indeed,
521 Colombo et al. [20] showed that geochemically-enriched icemelt is likely to be exported from the
522 RG towards the late snow-free season (August/September), after the winter snowpack melting and
523 prolonged periods of atmospheric temperature above 0 °C. Unfortunately, the lack of data on water
524 temperature, electrical conductivity and solutes concentration for 2016 does not allow to verify if
525 the described anomalies represent actual markers of RG influence emerging as a response to
526 stronger input events, rather than products of random interannual variability. Due to the limited
527 amount of information on this subject, we believe these observations may represent useful hints for
528 future investigations.
529
530 In conclusion, our results showed that different habitats of the Col d'Olen RG-pond system
531 harboured different, well separated archaeal and bacterial communities. They also suggest that the
532 presence of meltwater inputs from an active RG has only a limited influence on the abundance and
533 diversity of microbial communities inhabiting sediments of an adjacent water body. Indeed, no clear
534 evidence of a stable modification of microbial ecology or sediments geochemistry in the area
535 closest to the predicted RG inflow has been found. Rather, factors involved in stably shaping
536 microbial diversity and distribution in the overall pond system seem to be more linked to the
537 potential of the RG to supply new sedimentary material or to other dynamics intrinsic to the pond,
538 such as differences in water column depth and exposition to freeze-thaw processes.
539

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Figure Captions

Fig. 1 Location of the study area (a) and positioning of the sampling points (b, c) in the Col d'Olen Rock Glacier Pond and neighbouring compartments. In S1, S2 and S3 sediments and porewater were sampled and analysed. In RG and G fine material (0-10 cm depth) was sampled

Fig. 2 Geochemical properties (a-h), and abundance of 16S rRNA genes, quantified by qPCR for Archaea (i) and Bacteria (j) on RG, sediment (S1-S3) and soil (G) samples. In boxplots boxes represent the interquartile range between the first and third quartiles, whiskers indicate the range of measurements and bold bars show sample medians. In bar charts and dot plots bars and points correspond to sample means and error bars represent the standard error of the mean. Different colours correspond to different years (dark gray: 2015; light gray: 2016). Different letters indicate significant differences ($P < 0.05$) among samples collected in the same year assessed with Tukey HSD (a-g, i-j) or Dunn *post hoc* tests (h).

Fig. 3 Alpha diversity estimators calculated for archaeal (a-e) and bacterial (f-j) communities in the different sampling sites and times. Boxes represent the interquartile range between the first and third quartiles, whiskers indicate the range of measurements and bold bars show sample medians. Different letters indicate significant differences ($P < 0.05$) among samples collected in the same year assessed with Dunn's *post hoc* tests.

Fig. 4 NMDS ordination based on Bray-Curtis, Unweighted and Weighted UniFrac distance matrixes calculated using total archaeal and bacterial OTUs

Fig. 5 Relative abundance of the major archaeal (a) and bacterial phyla (b), with Proteobacteria split in classes, in the different sampling points and times. Each bar represents the average of three replicate samples. The group 'others' include all the taxonomic groups not explicitly reported in the legend

Fig. 6 Networks showing significant associations (IndVal > 0.90; $P_{FDR} < 0.05$) between different sampling points and archaeal (a) or bacterial (b) indicator OTUs assessed by indicator species analysis. For each group of associations, OTUs are represented as grouped in phyla/classes, and the actual number of OTUs is reported beside. The dimension of circles size is proportional to the number of representative OTUs included and the colour reflects the taxonomic affiliation. The length and thickness of the edges connecting OTUs and sampling points is not proportional to any evaluated parameter.

Figure 1

Click here to access/download;Figure;Fig1.tif

Figure 2

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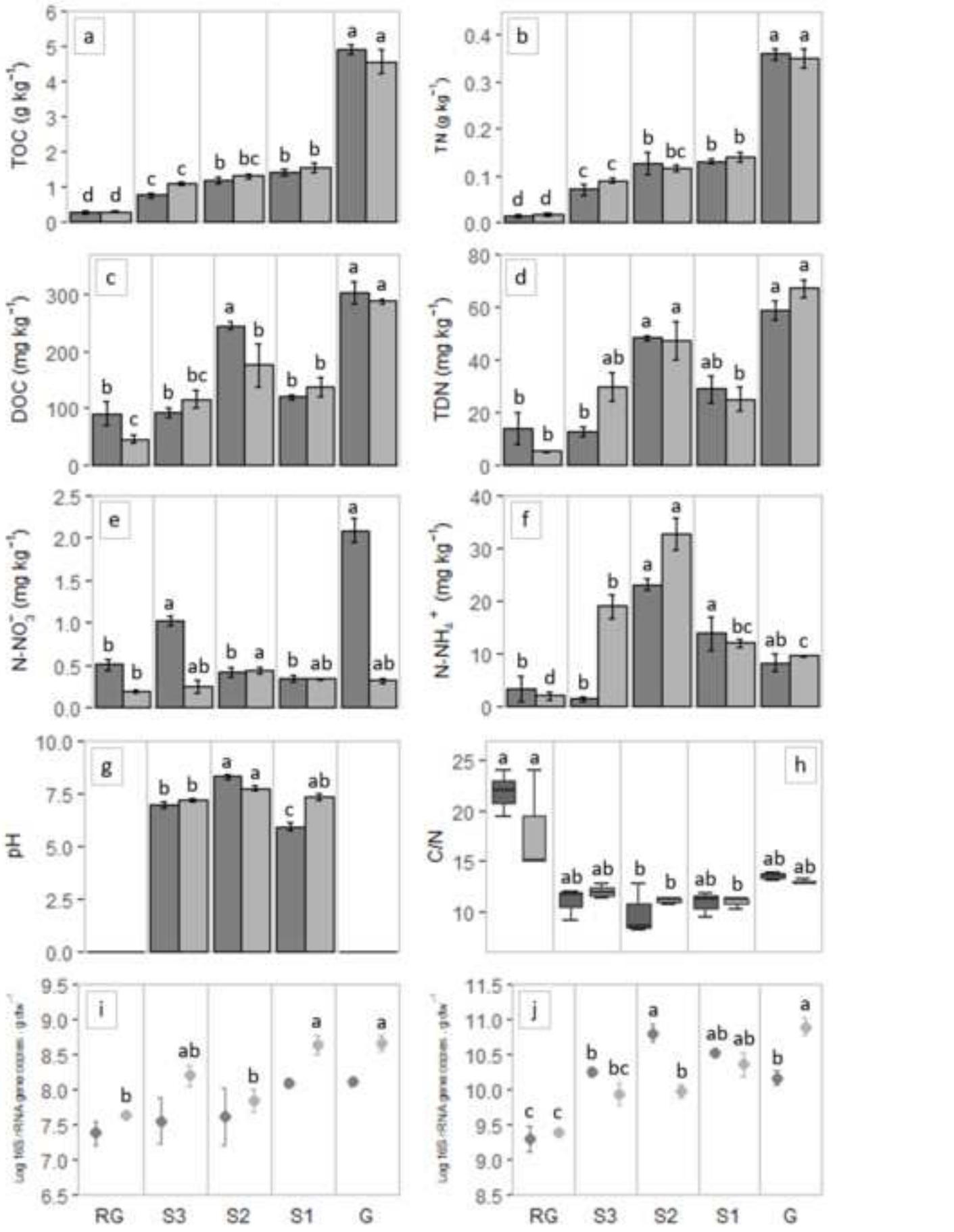

Figure 3

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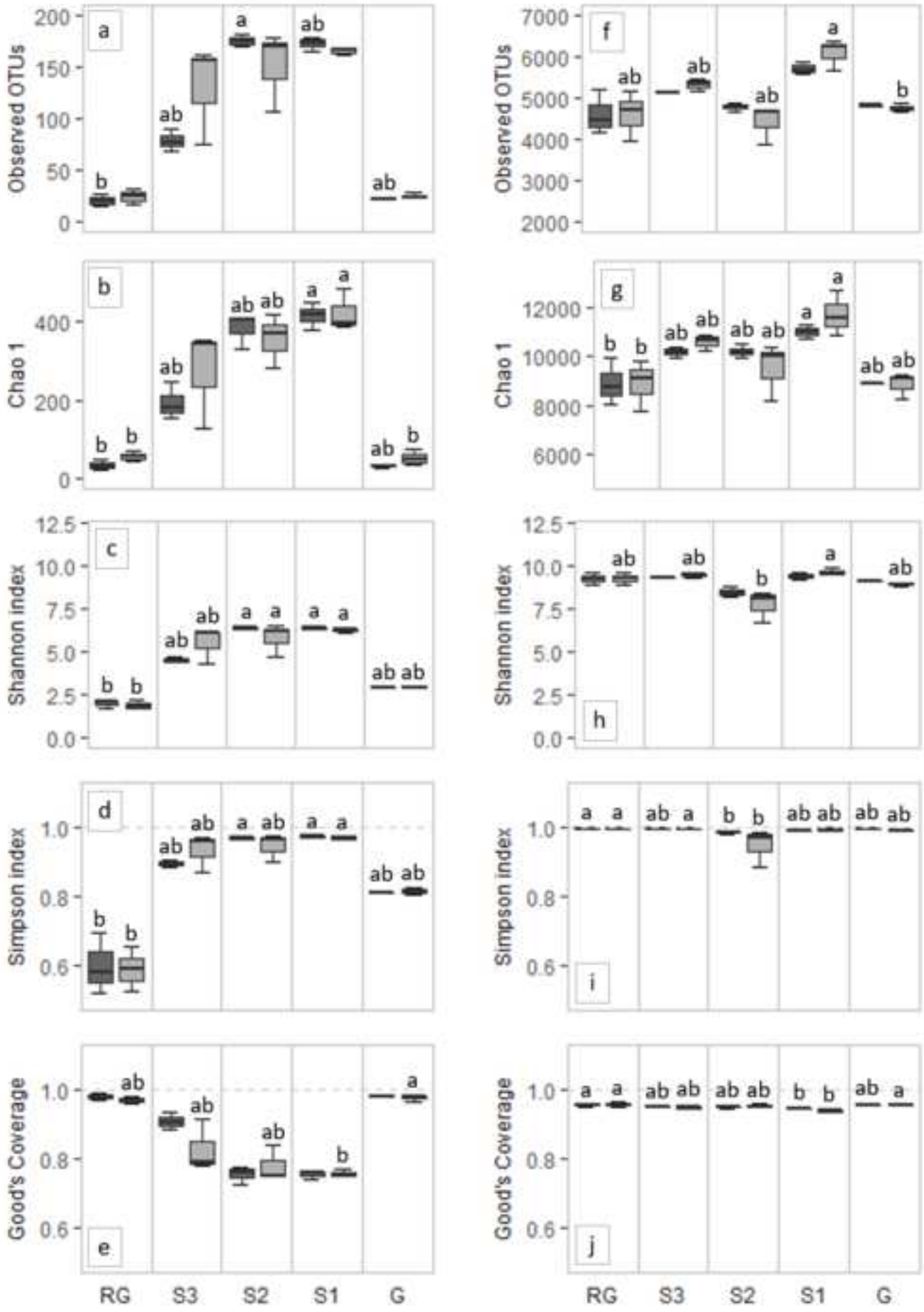

Figure 4

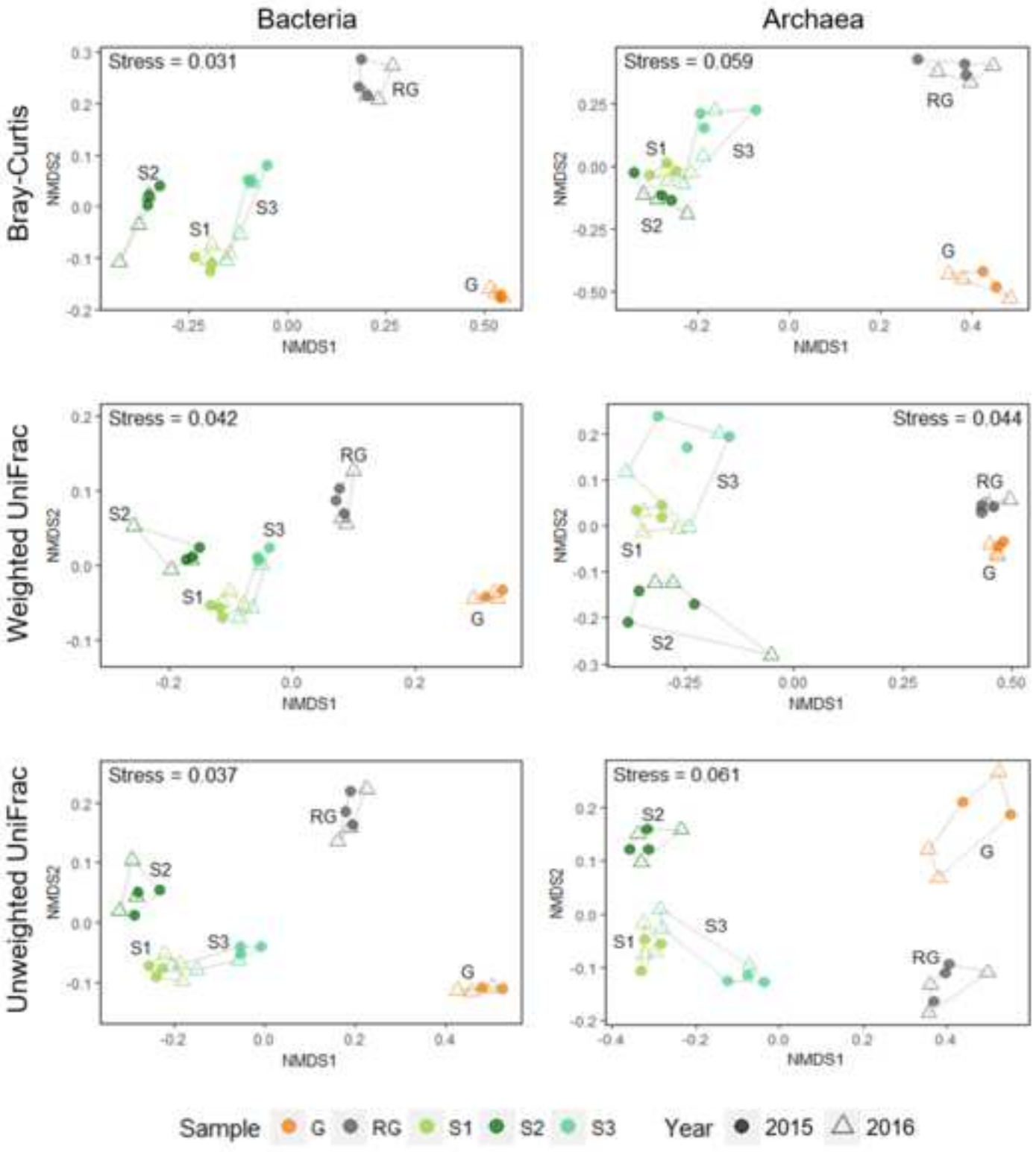

Figure 5

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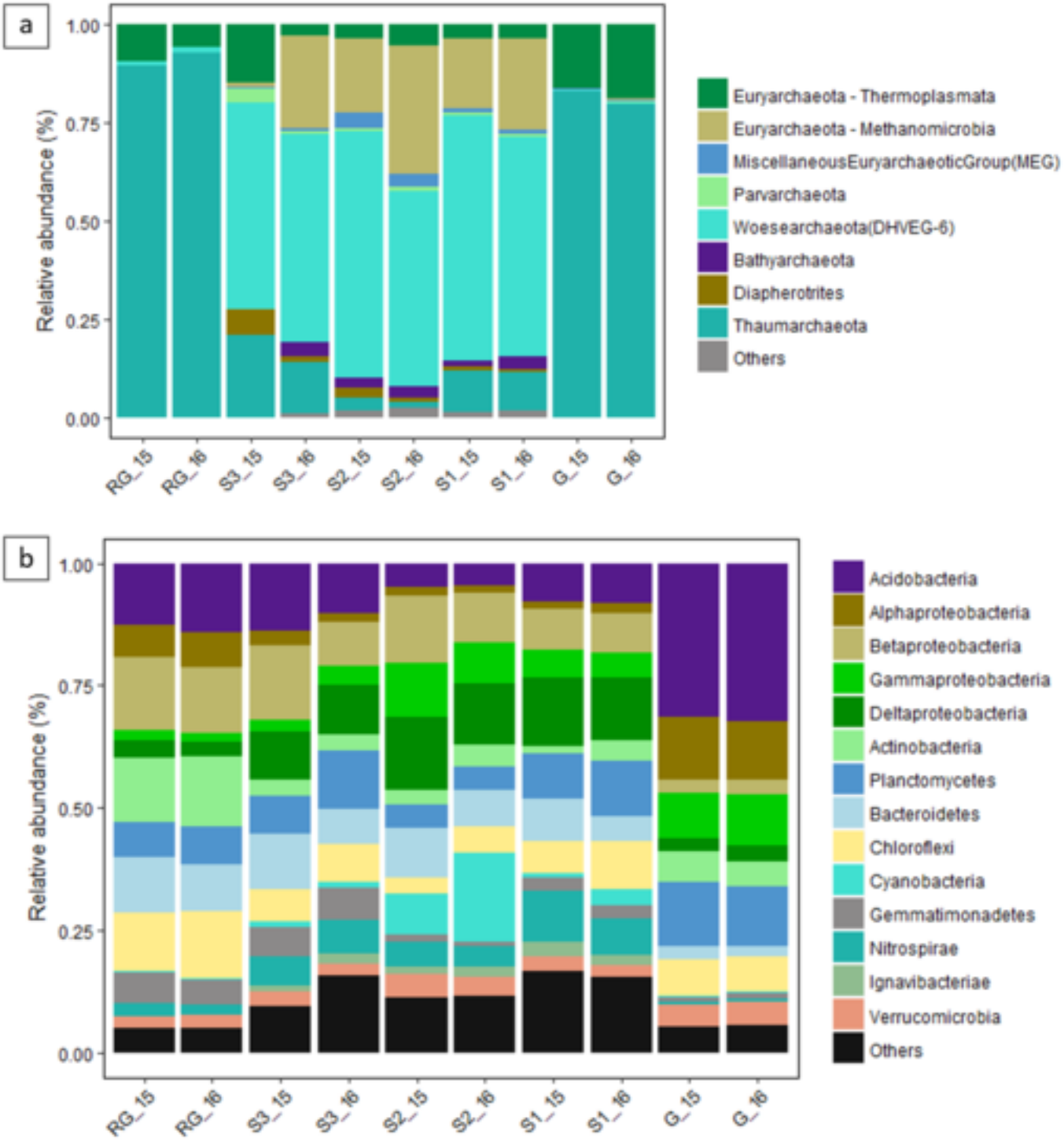

Figure 6

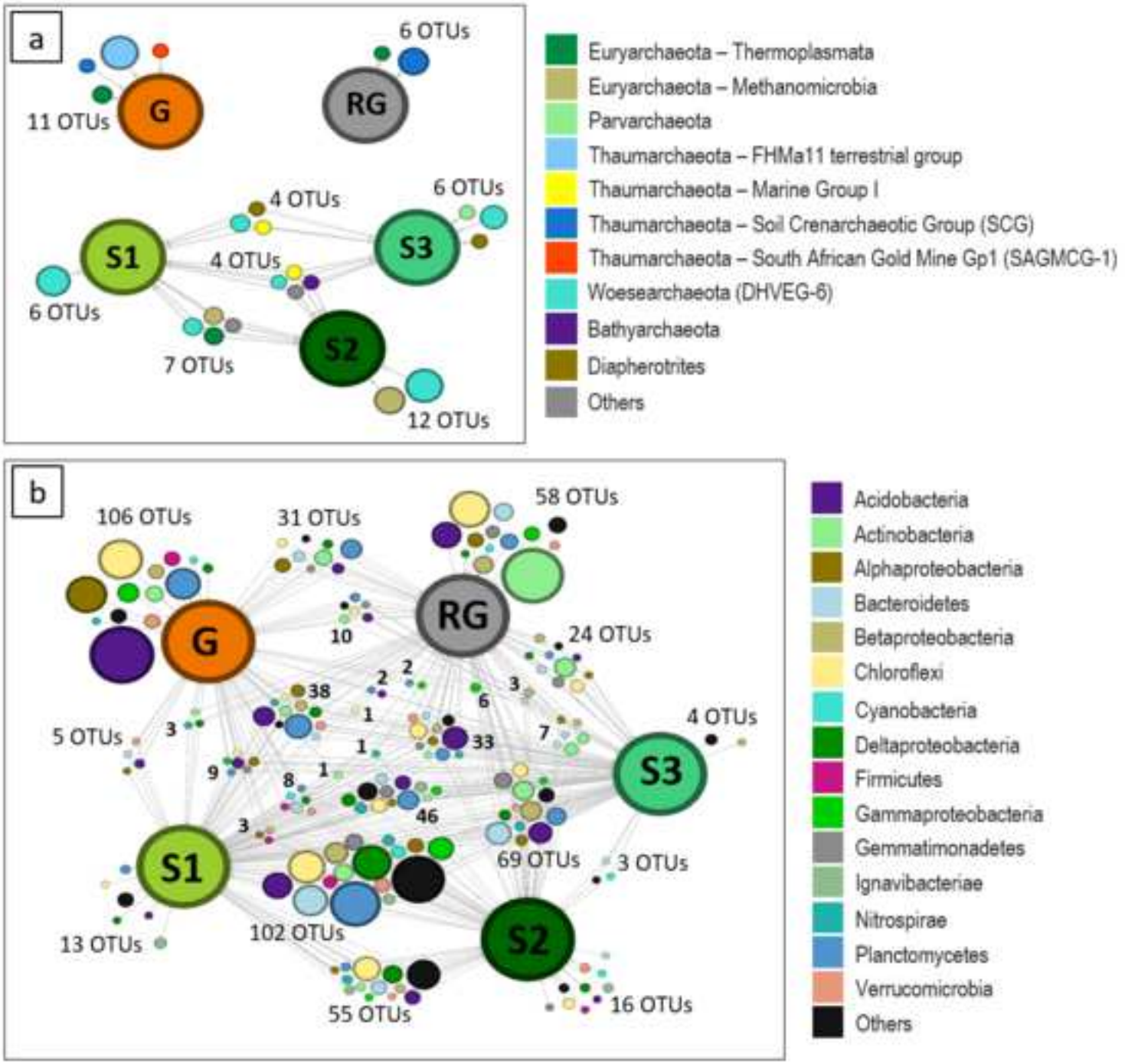

Table 1 Description of the sampling sites

Code	Environmental matrix	Dominant lithology	Depth (m)	n. of replicates
S1	Lacustrine sediment	Serpentinite	1	3
S2	Lacustrine sediment	Serpentinite	3	3
S3	Lacustrine sediment	Serpentinite	1	3
RG	RG fine grained surface material	Serpentinite	-	3
G	Soil	Calcschist	-	3

Table 2 Chemical characterisation of porewaters extracted from pond sediments. The maximum and minimum values measured for each year are reported

	2015	2016
pH	5.70 – 8.50	7.10 – 7.90
Si (mg L^{-1})	0.25 – 3.89	0.62 – 1.82
Na^{+} (mg L^{-1})	0.36 – 1.72	0.37 – 0.75
K^{+} (mg L^{-1})	0.96 – 4.60	1.01 – 4.52
Ca^{2+} (mg L^{-1})	5.61 – 50.9	2.66 – 14.7
Mg^{2+} (mg L^{-1})	2.50 – 22.4	1.02 – 4.02
Cl^{-} (mg L^{-1})	0.68 – 4.98	0.11 – 1.52
NO_2^{-} (mg L^{-1})	0.01 – 0.06	0.01 – 0.03
NO_3^{-} (mg L^{-1})	0.01 – 4.46	< 0.01 – 0.05
SO_4^{2-} (mg L^{-1})	1.67 – 11.4	< 0.01 – 17.6
PO_4^{3-} (mg L^{-1})	0.01 – 0.02	0.01 – 0.04